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The transmembrane tyrosine of μ-heavy chain is required for BCR destabilization and entry of antigen into clathrin-coated vesicles

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Abstract

The B cell antigen receptor (BCR) delivers antigen to the endocytic compartment and transduces signals that regulate the stability of the receptor complex. Previous studies showed that BCRmediated signal transduction dissociates μ-heavy chain (μm) from lg-α/lg-β, facilitating the delivery of antigen to clathrin-coated vesicles (CCVs). Herein, we demonstrate that the dissociation of lg-α/lg-β from mm requires tyrosine-587 of the μm transmembrane domain. Receptors expressing a mutation at tyrosine-587 (Y587F) transduced signals that were comparable to wild type, yet they failed to dissociate μm from lg-α/lg-β. Further, receptors harboring the Y587F mutation failed to associate with CCVs, resulting in diminished antigen in the lysosome-associated membrane protein-1 (LAMP-1^+) compartment and severely impaired antigen presentation, indicating that endocytosis through CCVs is required for antigen presentation. Thus, the transmembrane tyrosine of μm mediates destabilization of the BCR complex, facilitating antigen processing by promoting the association of antigen with CCVs.

Keywords

antigen processing/presentation; B cell; cell activation; signal transduction

Introduction

During T-dependent immune responses, the B cell antigen receptor (BCR) serves as a recognition receptor, allowing B cells to transduce antigen-mediated signals and endocytose receptors for antigen processing and presentation (1). Studies investigating the link between signal transduction and receptor endocytosis found that BCR-derived signals guide antigen-bound receptors to the MHC class II loading compartment (2). Antigen processing begins when receptors associate with clathrin-coated vesicles (CCVs) and are removed from the plasma membrane (3–6). Whether this process requires lipid rafts remains unclear (4–6). Antigen-bound receptors move to the late endosome, where protease degradation generates peptides that are loaded onto newly synthesized MHC class II molecules. Progression of antigen through the endocytic pathway requires BCR-derived signals that accelerate antigen degradation, direct remodeling and acidification of pre-existing endosomes and promote the formation of new endosomes (2,7–11). Although the BCR-derived signaling events that facilitate antigen processing within the endosome are becoming clear, the signals that

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promote the association of receptors with CCVs and initiate entry into the endocytic pathway remain poorly defined (5).

The BCR is composed of membrane Ig non-covalently associated with the lg-α/lg-β signal-transducing complex. Formation of the BCR complex requires interactions between the transmembrane tyrosine-587 and serine-588 of μ-heavy chain (μm) with undefined regions of lg-α/lg-β (12). Loss of polarity on these residues leads to the expression of unsheathed μm on the cell surface (13). Signal transduction through the BCR is initiated upon antigen-induced receptor aggregation. The cascades of events that result from protein-tyrosine kinase activation, phosphoinositide hydrolysis and calcium mobilization regulate gene transcription and cellular responses. One consequence of BCR-mediated signal transduction is the altered stability of the antigen- receptor complex. Antigen ligation reduces the amount of lg-α/lg-β that co-precipitates with μm (14). Electron microscopy studies showed that lg-α/lg-β physically dissociates from μm by distances approximating 180 nm (15). Destabilized lg-α/lg-β is found in lipid rafts in the absence of μm, while antigen-bound μm associates with CCVs in the absence of Ig-α (15). The coincidence of BCR destabilization and the association of unsheathed μm in CCVs suggested that the two events might be linked; however, the mechanism underlying the dissociation of lg-α/lg-β from μm is undefined.

Destabilization of the BCR complex leads to the dissociation of antigen-bound μm from the lg-α/lg-β signal-transducing complex. Early studies of BCR destabilization showed that pharmacologically inhibiting Src-family kinases prevented the dissociation of the receptor complex (14, 15). Interestingly, inhibiting Src kinases also prevented the association of antigen-bound μm with CCV (14, 15). This raised the possibility that the unsheathing of μm might be a requisite event for the entry of receptors into the endocytic pathway. However, inhibiting these signaling effectors might also inhibit the phosphorylation of clathrin heavy chain (5) or some other undefined function in endocytosis. To directly define if BCR destabilization was required for the entry of antigen into CCVs and to define if antigen processing and presentation occurred in the absence of clathrin-dependent endocytosis, we identified a BCR mutant that failed to dissociate lg-α/lg-β from μm upon receptor stimulation. We found that A20 cells expressing a BCR harboring a mutation within the transmembrane region of μm (Y587→F; Y/F) failed to dissociate lg-α/lg-β from μm, coincident with failed association of antigen-bound μm with CCVs. In addition, the Y/F mutation inhibited entry of antigen into late endosomes and markedly diminished the presentation of processed peptide to T cells. These data definitively established a role for BCR destabilization in antigen processing. Further, since the μm Y/F mutation does not disrupt movement of the mutated receptors to lipid rafts (16), yet antigen processing is severely impaired, the data indicate that BCR-derived antigens enter the endocytic pathway predominantly through CCVs rather than lipid rafts.

Methods

Cells

A20 murine lymphoma cells (lgG2a$^+$, H-2^d) transfected with phosphorylcholine (PC)-specific human wild-type (WT), Y587F mutant (Y/F), or Y587V S588V (YS/VV) IgM receptors were maintained as described (17). To directly compare A20WT with A20Y/F cells, we sorted the A20Y/F and A20YS/ VV cells to match the BCR expression levels of A20WT. The T cell hybridoma, DO11.10, is specific for ovalbumin peptide ($OVA_{323–339}$) bound to I-A^d (18).

Antibodies and reagents

Gold-labeled antigens and antibodies for immunoblotting, immunofluorescence and immunogold labeling were previously described (15). Rat anti-lysosome-associated membrane protein-1 (LAMP-1) was kindly provided by Marcus Clark (University of Chicago, IL, USA). The rabbit polyclonal anti-Syk was prepared against residues 257–352 of the murine Syk linker region (14, 19) and provided by John Cambier (University of Colorado Health Science Center, CO, USA). The rabbit polyclonal anti-lg-α was prepared against residues 160–220 of the mouse lg-α cytoplasmic tail (14, 19) and provided by John Cambier (University of Colorado Health Science Center). The rabbit antiserum was purified using peptide antigen coupled to sepharose and specificity was confirmed by immunoprecipitation and immunoblotting cell lysates and recombinant proteins. The monoclonal anti-Ig-β (HM-79) recognizes the extracellular domain of murine lg-β and has been previously described (20). Goat antihuman IgM was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and anti-Erk1/2 from Cell Signaling (Danvers, MA, USA). Rat anti-mouse MHC class II (M5/114) was used to measure the level of $I\text{-}A^d$.

Immunoprecipitation and surface biotinylation

Cells were immunoprecipitated with CNBr-activated Sepharose 4B-conjugated antibodies as described (15). Cells were surface biotinylated with EZ-Link™Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) as described (14).

Preparation of plasma membrane sheets and immunogold labeling for transmission electron microscopy

Preparation of plasma membrane sheets has been described (15). The criteria used to assess the association of μm and lg-α/lg-β was previously established in the IgM/α chimeric cell line. This receptor contains the NP-specific extracellular domain fused to Ig-α cytoplasmic tail (lgM/α). The distance between μm and Ig-α on this receptor averages 11 nm (15). To be conservative, 10- and 5-nm gold particles separated by >30 nm were considered dissociated. The association of μm with CCVs was defined by the presence of 10-nm gold particles (μm) in vesicles that stained specifically for clathrin (5 nm), and that exhibited a prototypic polyhedral structure.

Immunofluorescence staining

Cells were stained for μm and Ig-β as described (15). Briefly, the BCR complex was labeled on ice with fluorochromeconjugated PC-BSA or biotinylated HM-79 (anti-lg-β), followed by fluorochrome-conjugated streptavidin. Cells were incubated at 37°C for 30 min to allow endocytosis. After fixation with 3% PFA, cells were permeabilized with 0.05% saponin and stained for LAMP-1.

Antigen presentation assay

A20 transfectants were pulsed with PC3–OVA (30 μg ml^{-1}) or OVA antigen (300–1000 μg ml^{-1}) for 24 .h, fixed with 1% PFA and incubated with DO11.10 cells (30 000) for 24 h as described (17). IL-2 secreted from T cells was measured by ELISA.

Results

Our previous work showed that pharmacological inhibitors that prevent BCR destabilization preclude the association of antigen-bound receptors with CCVs (15). To directly test the hypothesis that BCR destabilization was required for the association of receptors with CCVs, we sought to identify a BCR mutant that sheathes lg-α/lg-β, but fails to undergo

destabilization upon receptor ligation. We targeted amino acids within the μm transmembrane region because antigen stimulation of orthophosphate-loaded cells showed a 2-fold increase in phosphate incorporation within μm (data not shown). Given that the cytoplasmic tail of μm does not contain amino acids modified by phosphorylation, we reasoned that the 'transmembrane' region was involved since five out of the eight residues proximal to the cytoplasmic tail are serine, threonine or tyrosine. Previously, cell lines harboring site-specific mutations within the transmembrane region were used to define the residues required in the sheathing of μm with lg-α/lg-β (17, 21, 22). Among the mutants that sheathe lg-α/lg-β, we first tested whether Y587 was necessary for the dissociation of lg-α/lg-β from μm. As shown in Fig. 1(A), the expression of human PC-specific WT IgM and mutant Y587 (Y/F) receptors was comparable. To assess if the dissociation of lg-α/lg-β from μm was evident following antigen stimulation, we quantitated the amount of lg-α/lg-β associated with surface μm by immunoprecipitating biotinylated surface WT and Y/F receptors. A20WT cells stimulated with PC-BSA showed a 2-fold decrease in the amount of co-precipitated Ig-α (Fig. 1B, streptavidin immunoblot). This is consistent with previous work showing that antigen stimulation of the BCR leads to a destabilization of the lg-α/lg-β signal-transducing complex (14, 15). In contrast, antigen stimulation of A20Y/F cells failed to decrease the amount of surface Ig-α associated with μm. This suggests that tyrosine-587 of μm controls the destabilization of lg-α/lg-β from μm. It was somewhat surprising that despite a 2-fold reduction in the amount of lg-α co-precipitated with surface μm (Fig. 1B, streptavidin immunoblot), this reduction was not evident when lg-α levels were quantitated from the total BCR pool (Fig. 1B, anti-Ig-α immunoblot). We reasoned that this might be due to a large pool of intracellular receptors that were not exposed to antigen and therefore remained associated with lg-α/lg-β. This pool of receptors might mask the presence of dissociated receptors if the membrane pool was significantly smaller. To resolve this discrepancy, we separated the surface receptor pool from the cytoplasmic pool using surface biotinylation. The cell lysates from unstimulated and antigen-stimulated cells were divided and the total BCR pool was immunoprecipitated with anti-μ (Fig. 1C; lanes 1 through 4) while the cytoplasmic pool was isolated by streptavidin depletion (lanes 5 and 6). Lanes 3 and 4 (total membrane + cytoplasmic BCR) showed that the intracellular pool of receptors was significant in this cell line. As expected, this pool of receptors did not exhibit BCR destabilization (Fig. 1C; lanes 5 and 6). In contrast, the membrane pool (Fig. 1C; streptavidin immunoblot, lanes 1 and 2) revealed significantly reduced level of Ig-α associated with membrane μm following antigen stimulation. Collectively, the data show that in some cell lines, a large cytoplasmic pool of BCR obscures BCR destabilization in the absence of isolating surface receptors exposed to antigen. Nonetheless, the A20WT cells dissociate Ig-α following antigen stimulation as previously described for the K46μ, M12g3r cell lines and primary B cells from 3–83μδ mice (14, 15). In contrast, mutation of μm transmembrane Y587 abolishes BCR destabilization.

Analysis of native membranes by transmission electron microscopy (TEM) has been used to identify receptor domains and to show the dissociation of Ig-α from μm (15, 23). To further investigate the possibility that A20Y/F cells failed to dissociate lg-α/lg-β, we assessed their localization on native membrane sheets prepared from A20WT and A20Y/F cells. Resting A20WT and A20Y/F cells stained with gold-labeled PC_{10}–BSA (10 nm) and Ig-α (5 nm) showed 51 and 52% colocalization of μm and Ig-α (Fig. 2A and C and Table 1). This level of co-localization was consistent with our previous study where immunogold staining of μm and lg-α from a chimeric receptor (μm/lg-α) approximated 50% co-localization, likely due to the inability to label the BCR at saturating levels (15). Within 5 min of stimulation, A20WT cells reduced the co-localization of μm with Ig-α to 25% with distances of 160 nm separating the receptor subunits (Fig. 2B and Table 1). In contrast, μm and lg-α expressed on the A20Y/F cells remained co-localized (54%) following 5 and 10 min of antigen stimulation (Fig. 2D and Table 1). The staining was specific in that gold-BSA (Fig. 2E),

isotype matched, unrelated antibodies and secondary antibodies (Fig. 2F) failed to stain the membranes. These data reveal a site within μm that is targeted to dissociate lg-α/lg-β from lm during receptor de-stabilization.

The dissociation of lg-α/lg-β from μm requires the activation of BCR-mediated signal transduction. Although the identity of the kinase(s) remains unknown, we have shown that inhibiting the activation of Lyn and Syk prevents BCR destabilization (14, 15). We reasoned that the A20Y/F cell line might fail to dissociate lg-α/lg-β from μm because the Y587 mutation delayed the kinetics or decreased the magnitude of antigen-induced signal transduction. Although others showed that the release of intracellular calcium from A20WT and A20Y/F cells was comparable (17), it was possible that calcium release might not impact the stability of the BCR complex but that other signaling pathways critical in BCR destabilization might be affected. To investigate this, we assessed BCR-induced phosphorylation of lg-α, Lyn, Syk and Erk. As shown in Fig. 3, after 1 and 5 min of antigen stimulation, the levels of Ig-α, Lyn, Syk and Erk phosphorylation was comparable. Given that destabilization of lg-α/lg-β occurred at these times (Fig. 2), our data indicate that mutation of Y587 fails to dissociate lg-α/lg-β from μm despite intact BCR-mediated signaling. These findings are consistent with a direct role for Y587 in BCR destabilization (17).

Our previous studies showed that lg-α/lg-β dissociated from μm and suggested that BCR destabilization attenuated BCR-mediated signal transduction (14, 19); however, the biological significance of this phenomenon has remained unclear. Further, the findings that 91% of receptors within CCVs were unsheathed (15) suggest that BCR destabilization occurs prior to the association of antigen-bound μm with the CCVs. Whether destabilization of the receptor was coincidental or a required event for the antigen association with CCVs was unknown. To test whether receptors that remained competent to transduce signals, yet failed to dissociate μm from lg-α/lg-β, were capable of entering CCVs, we enumerated receptors associated with CCVs in the presence or absence of antigen stimulation. In unstimulated A20WT and A20Y/F cells, 7 or 8% of μm (10 nm) was associated with clathrin (5 nm) (Fig. 4A and C and Table 1). Stimulation of A20WT cells induced a 3.5-fold increase in the association of μm with CCVs within 10 min (Fig. 4B and Table 1). In contrast, stimulation of A20Y/F cells failed to induce the association of μm with CCVs (Fig. 4D and Table 1). This suggests that although the Y587F mutation does not disrupt BCR-mediated signal transduction (Fig. 3), it fails to dissociate lg-α/lg-β from μm (Figs 1 and 2) and fails to promote association of antigen-bound μm with CCVs (Fig. 4). This indicates that BCR-derived signals target the trans-membrane tyrosine of μm to destabilize the receptor complex and facilitate the entry of antigen-bound μm into CCVs.

The data suggest that the unsheathing of μm is required for the association of antigen-bound receptors with CCVs. To investigate if the dissociation of lg-α/lg-β from μm was sufficient to promote CCV association, we enumerated the localization of YS/VV receptors with CCVs (Table 2). The YS/ VV cell line creates a BCR that is expressed on the cell surface in the absence of lg-α/lg-β (24). As shown in Fig. 5(A), the expression level of the human PC-specific receptor on A20YS/VV cells was slightly lower than receptor expression on either A20WT or A20Y/F cells, consistent with previous observations (13, 25). We reasoned that if dissociation of lg-α/lg-β were sufficient to promote the association of μm with clathrin, these receptors would constitutively localize within clathrin cages. As shown in Fig. 5 and Table 2, TEM analysis showed that the YS/VV receptors were not associated with CCV in the presence or absence of receptor aggregation. Despite the slightly reduced expression of the YS/VV receptor (35% reduced compared with WT), the staining on the membrane sheets was consistently increased (density measure of gold particles /cm^2; 0.7 in Fig. 5B

versus 2 in Fig. 5D). Nonetheless, the data indicate that simple unsheathing of μm does not promote its association with CCVs.

Endocytosis of antigen-bound receptors leads to antigen processing, degradation or receptor recycling. Although controversial, receptor internalization through lipid rafts has been shown to mediate antigen processing (6, 26). Our data indicate that although the A20Y/F cell line transduces BCRmediated signals, it fails to associate with CCVs (Fig. 4); however, the A20Y/F receptor associates with lipid rafts (16). This raised the possibility that in the absence of clathrinmediated endocytosis, lipid raft-mediated endocytosis could facilitate antigen presentation. To assess if blocking clathrinmediated endocytosis affected antigen processing, we monitored the accumulation of fluorochrome-tagged antigen and Ig-β in the LAMP-1^+ late endosomes following antigen stimulation (Fig. 6A and B). In unstimulated A20WT and A20Y/F cells, the majority of LAMP-1 staining was intracellular and the BCR was localized on the cell surface (Fig. 6A and B, upper panels). Following stimulation of A20WT cells, antigen co-localized with the intracellular LAMP-1^+ compartments; however, Ig-β remained on the cell surface (Fig. 6A and B, lower panels of A20WT), consistent with the idea that BCR ligation destabilizes lg-α/lg-β from μm and that unsheathed μm targets antigen to the endocytic pathway. In contrast, stimulation of A20Y/F cells failed to promote antigen localization into the LAMP-1^+ compartments (Fig. 6A, lower panel of A20Y/F). However, the staining of antigen and Ig-β from the A20Y/F cells was intracellular, indicating that receptors harboring the Y587F mutation internalized, but did not traffic into late endosomes (Fig. 6A and B, lower panels of A20Y/F). This suggests that in the absence of BCR destabilization, antigen processing is impaired, yet receptors clear the membrane. To confirm that antigen in the LAMP-1^+ compartments reflected cell surface presentation of antigen, we quantitated the amount of IL-2 secreted by T cells upon recognition of processed peptides presented by MHC class II molecules. A20WT cells pulsed with PC_3–OVA induced secretion of IL-2 by $OVA_{323–339}$-specific DO11.10 T cells (Fig. 6C). In contrast, A20Y/F cells pulsed with PC_3–OVA failed to induce IL-2 secretion. This did not reflect changes in surface MHC class II expression (Fig. 6D) or overall defects in antigen processing because presentation of OVA internalized by pinocytosis was comparable between A20WT and A20Y/ F cells (data not shown). The data indicate that in the absence of BCR destabilization and association of antigen-bound μm with CCVs, antigen processing and presentation are attenuated. This is consistent with the failure of antigen to reach the LAMP-1^+ compartments (Fig. 6A). Thus, the transmembrane tyrosine of μm is required in antigen processing and presentation because it plays a critical role in facilitating BCR destabilization. Further, destabilization is required for the association of antigen-bound μm with CCVs, an event that cannot be substituted by lipid raft-mediated endocytosis.

Discussion

Antigen binding to the BCR induces destabilization of the receptor complex, an event that physically dissociates lg-α/lg-β from μm. BCR destabilization is evident in biochemical and cellular fractionation studies and by confocal and electron microscopy (14, 15). Biochemical studies show that immunoprecipitation of antigen-stimulated BCR complexes fails to co-precipitate stoichiometric amounts of lg-α/lg-β from cell lines and primary splenic B cells expressing Ig transgenes. Further, fractionation by sucrose gradient centri-fugation co-localizes Ig-α with markers of lipid rafts in the absence of μm (15). On a cellular level, confocal microscopy studies show that in antigen-stimulated cells, Ig-β fails to co-localize within μm-induced caps and Ig-α fails to co-localize with antigen in the LAMP-1^+ compartment (15) (Fig. 6A and B). Further, others showed that μm and lg-α/lg-β are independently internalized after antigen stimulation (27). Lastly, TEM analysis of native membranes shows the separation of μm and Ig-α by >180 nm and the association of antigen-bound μm with CCVs in the absence of Ig-α (15). One study reports that BCR

destabilization is not evident by FRET in a J558L cell line (28). Given that the signaling pathways required for BCR destabilization have not been defined, this discrepancy might reflect impaired calcium mobilization in the J558 cell line since this plasmacytoma lacks CD19, an essential co-receptor for calcium responses (29–33).

Until this study, the biological consequences of BCR destabilization have remained unclear. The initial work defining BCR destabilization as a consequence of BCR ligation suggested that it might play a role in desensitizing or inducing unresponsiveness to self-antigen. Indeed, the unresponsive phenotype of Smith antigen (Sm)-specific B cells (34) is coincident with receptor destabilization; however, direct proof that destabilization is required in B cell tolerance will require genetic manipulation (M. Borrero and B. Vilen, unpublished observations). Our recent study suggests a role for BCR de-stabilization in the association of antigen-bound receptors with CCVs and antigen processing/presentation (15). This idea came from our findings that pharmacological inhibitors prevented the association of μm with CCV coincident with inhibiting BCR destabilization. This indicates that receptor destabilization might be required during CCV-mediated en-docytosis. However, it was argued that pharmacological manipulation might disrupt other critical events required for the entry of receptors into CCVs. Thus, a genetic approach was necessary to directly assess the role of BCR destabilization in clathrin-mediated endocytosis. In this study, we identified a mutation within the μm transmembrane region that was required for BCR destabilization. Mutation of tyrosine-587 maintained the association of μm with lg-α/lg-β and allowed antigen-induced signal transduction (17) (Fig. 3), yet failed to dissociate μm from lg-α/lg-β (Figs 1 and 2). These findings allowed us to directly assess if BCR destabilization was required for the entry of antigen-bound μm into the endocytic pathway via CCVs. The data showed that receptors failing to undergo receptor destabilization failed to associate with CCVs (Fig. 4) and to process or present antigen to T cells (17) (Fig. 6). Collectively, these studies identify the transmembrane tyrosine of μm as playing a key role in receptor destabilization and show that it plays a central role in the association of antigen-bound receptors with CCVs.

The exact mechanism by which BCR destabilization promotes the association of antigen-bound receptors with CCVs remains unclear. Our previous data indicated that >90% of receptors within CCVs were unsheathed (15). Since these receptors lack lg-α/lg-β the data indicate that association of antigen-bound μm with CCVs occurs by a mechanism other than the classical YXXΦ sorting motif (35, 36). One possibility is that BCR destabilization reveals a novel endocytosis motif that is masked when lg-α/lg-β associated with μm. However, analysis of the A20YS/VV cell line failed to show constitutive association of unsheathed, YS-mutated receptors with CCVs (Fig. 5E). This indicates that exposure of the transmembrane region alone is insufficient to induce the association of receptors with CCVs and that BCR destabilization does not simply expose a unique endocytosis motif. Another possible mechanism is that BCR-mediated signal transduction modifies the transmembrane tyrosine, thereby dissociating lg-α/lg-β from μm. For this scenario, the transmembrane amino acids that are shielded by lg-α/lg-β (within the carboxy-terminal polar patch) must become exposed to cytoplasmic kinases. Others have suggested that antigen ligation of the BCR induces conformational changes that displace μm into the cytoplasm or create a rotational torque on μm (37, 38). These modifications might induce the dissociation of lg-α/lg-β and provide a docking site for a chaperone, or clathrin adaptor molecule on μm.

BCR destabilization and the association of receptors with CCVs are dependent on the activation of the Src-family kinase, Lyn (14, 15). Others have described that entry of antigen into CCVs involves Lyn-mediated phosphorylation of clathrin heavy chain (5). Our data indicate that despite normal Lyn phosphorylation (Fig. 3), receptors failed to dissociate lg-α/lg-β from μm and failed to associate with CCVs. This suggests that clathrin heavy-chain

phosphorylation is not sufficient for the entry of antigen-loaded μm into CCVs. To assess if clathrin heavy-chain phosphorylation was evident in A20WT and A20Y/F cells following Lyn activation and thereby establish if BCR destabilization or clathrin heavychain phosphorylation was the initiating event in receptor association with CCVs, we immunoprecipitated clathrin heavy chain and immunoblotted for phosphotyrosine. We were unsuccessful in detecting clathrin phosphorylation in A20WT cells despite confirming that human B cells show moderate antigen-induced clathrin phosphorylation as previously reported (data not shown) (5). Thus, we propose a model wherein antigen-induced signal transduction activates Lyn and possibly prepares CCVs for entry of antigen-bound receptors through the phosphorylation of clathrin heavy chain. Simultaneously, antigen-mediated signal transduction induces BCR destabilization and possibly modifies μm to facilitate entry of antigen-bound receptors into CCVs. In the absence of destabilization, antigen-bound receptors do not enter the endocytic pathway as evidenced by the failure to detect antigen in the LAMP-1^+ compartments and the failure to present antigens to T cells. In summary, BCR destabilization is required for association of antigen with CCVs; however, the role of clathrin heavy-chain phosphorylation remains unclear since it is undetectable in our murine cell line.

The inability of μm to associate with clathrin markedly reduced the co-localization of antigen and LAMP-1 and the secretion of IL-2 by antigen-specific T cells. This indicates a marked defect in antigen processing and presentation and implies that clathrin-mediated endocytosis is the primary mode of antigen entry that promotes antigen processing/ presentation. The contribution of lipid raft-mediated versus clathrin-mediated endocytosis in the targeting of antigen to the endocytic pathway has been controversial, mainly because these studies involved genetic or pharmacological manipulation (5, 6). Studies have shown that although eliminating clathrin heavy chain diminishes receptor-mediated endocytosis, it also diminishes fluid-phase endocytosis and receptor recycling (39). Similarly, disrupting lipid rafts with methyl-β-cyclodextrin blocks the aggregation of lipid rafts, but also impairs the phosphorylation of clathrin heavy chain and other BCR-dependent functions (5, 40). In this study, we show that failure of μm to associate with CCVs markedly hindered antigen presentation (Fig. 6C) under conditions where clathrin and lipid rafts were not genetically or pharmacologically altered. Although it remains unclear whether clathrin that is raft associated (5) or clathrin that is membrane resident (4, 26) represents the pool for receptor endocytosis, our data demonstrate that the presentation of antigen via the BCR requires clathrin-mediated endocytosis.

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Abbreviations

μm	μ-heavy chain
BCR	B cell antigen receptor
CCV	clathrin-coated vesicle
FRET	Fluorescence Resonance Energy Transfer

IP	Immunoprecipitation
LAMP-1	lysosome-associated membrane protein-1
OVA	ovalbumin peptide
PC	phosphorylcholine
TEM	transmission electron microscopy
WCL	Whole Cell Lysate
WT	wild type

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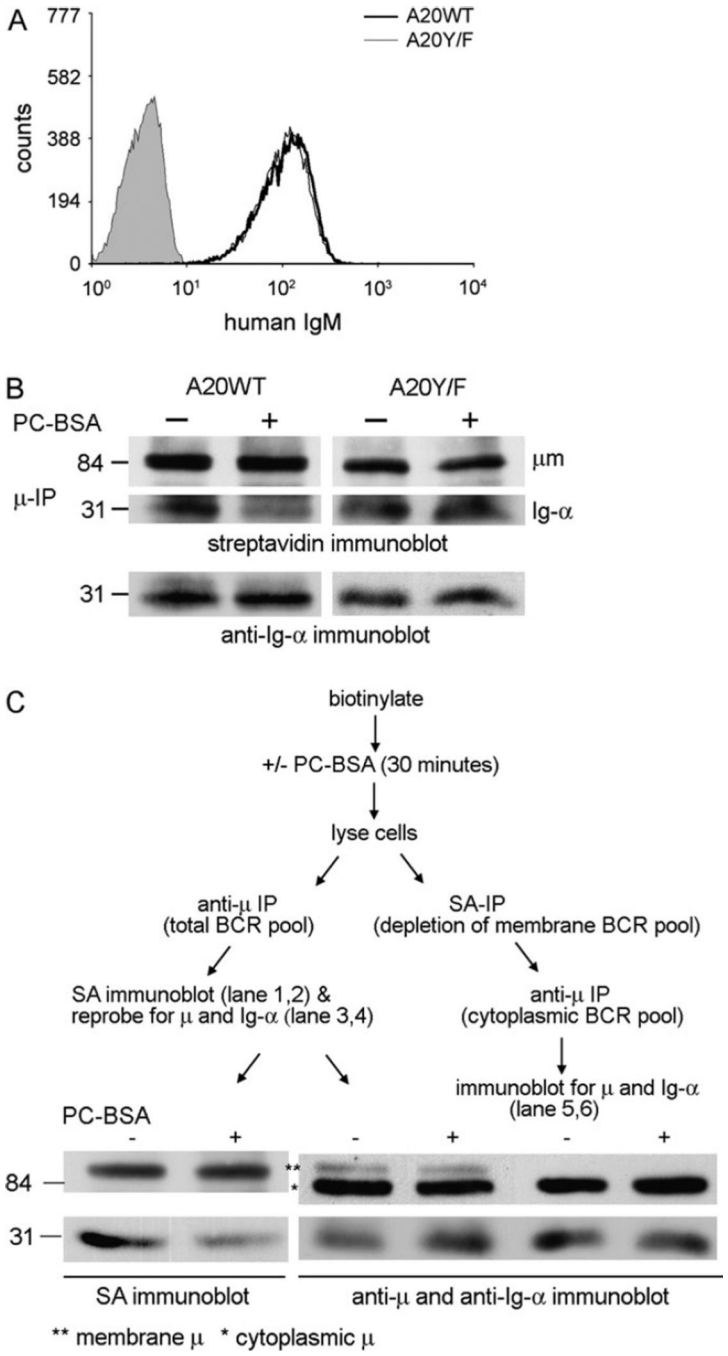

Fig. 1.

Mutation of the transmembrane tyrosine of μm inhibits BCR destabilization. (A) A20WT (thick line) and A20Y/F (thin line) cells were stained with FITC-conjugated anti-human IgM and analyzed by flow cytometry. The filled histogram represents cells stained with isotype control antibody. (B) Surface-biotinylated cells (30×10^6 cells) were either unstimulated or antigen stimulated for 30 min. The BCR complex was immunoprecipitated and immunoblotted with streptavidin–HRP, followed by anti-Ig-α. The data are representative of five experiments. (C) Surface-biotinylated cells (30×10^6 cells) were either unstimulated or antigen stimulated for 30 min. The BCR complex was immunoprecipitated and immunoblotted with streptavidin–HRP (lanes 1 and 2), followed by anti-μ and Ig-α (lanes 3

and 4). Simultaneously, the biotinylated surface BCR pool was depleted by streptavidin immunoprecipitation and the intracellular BCR pool was immunoprecipitated, followed by immunoblotting with anti-μ and Ig-α (lanes 5 and 6). The data are representative of five experiments.

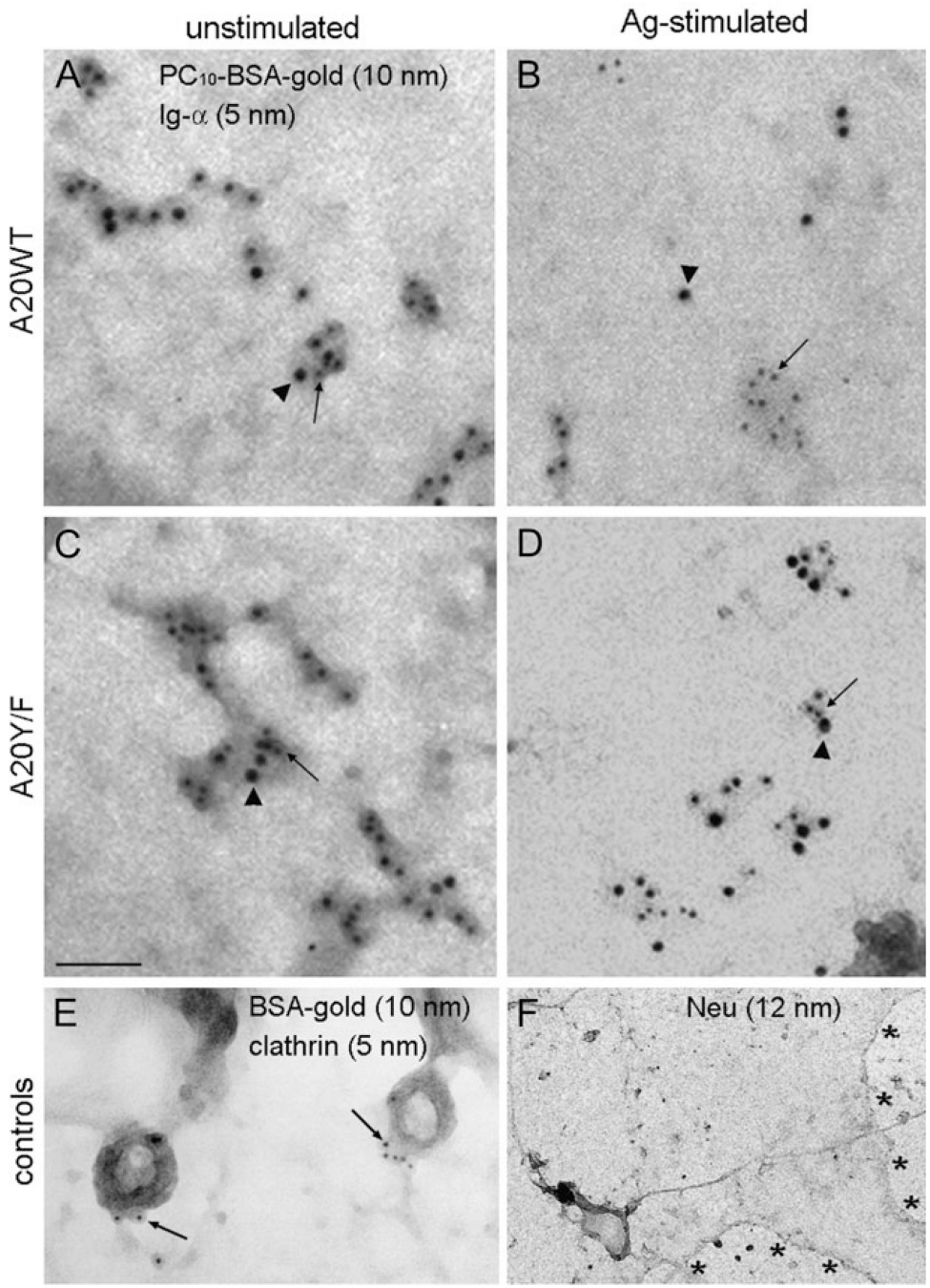

Fig. 2.

The μm harboring a Y587 mutation fails to dissociate Ig-α upon antigen stimulation. (A–D) Cells were either fixed prior to staining for μm or stimulated with PC_{10}–BSA–gold (10 nm) for 10 min. Native plasma membranes were prepared and stained for Ig-α (5 nm). (E) Cells were stained with BSA–gold (10 nm) and anti-clathrin (5 nm) or (F) an isotype matched, unrelated primary (anti-Neu) and secondary antibodies (12 nm) after preparing native membrane sheets. TEM images were taken at a magnification of ×25 000. The numbers of 10-nm gold particles are tabulated in Table 1 and represent data from 12 different native membrane sheets representing three experiments. Arrowheads and small arrows indicate

antigen (10 nm) and Ig-α (5 nm) or clathrin (5 nm) staining, respectively; *indicates the membrane edge. Bar = 100 nm.

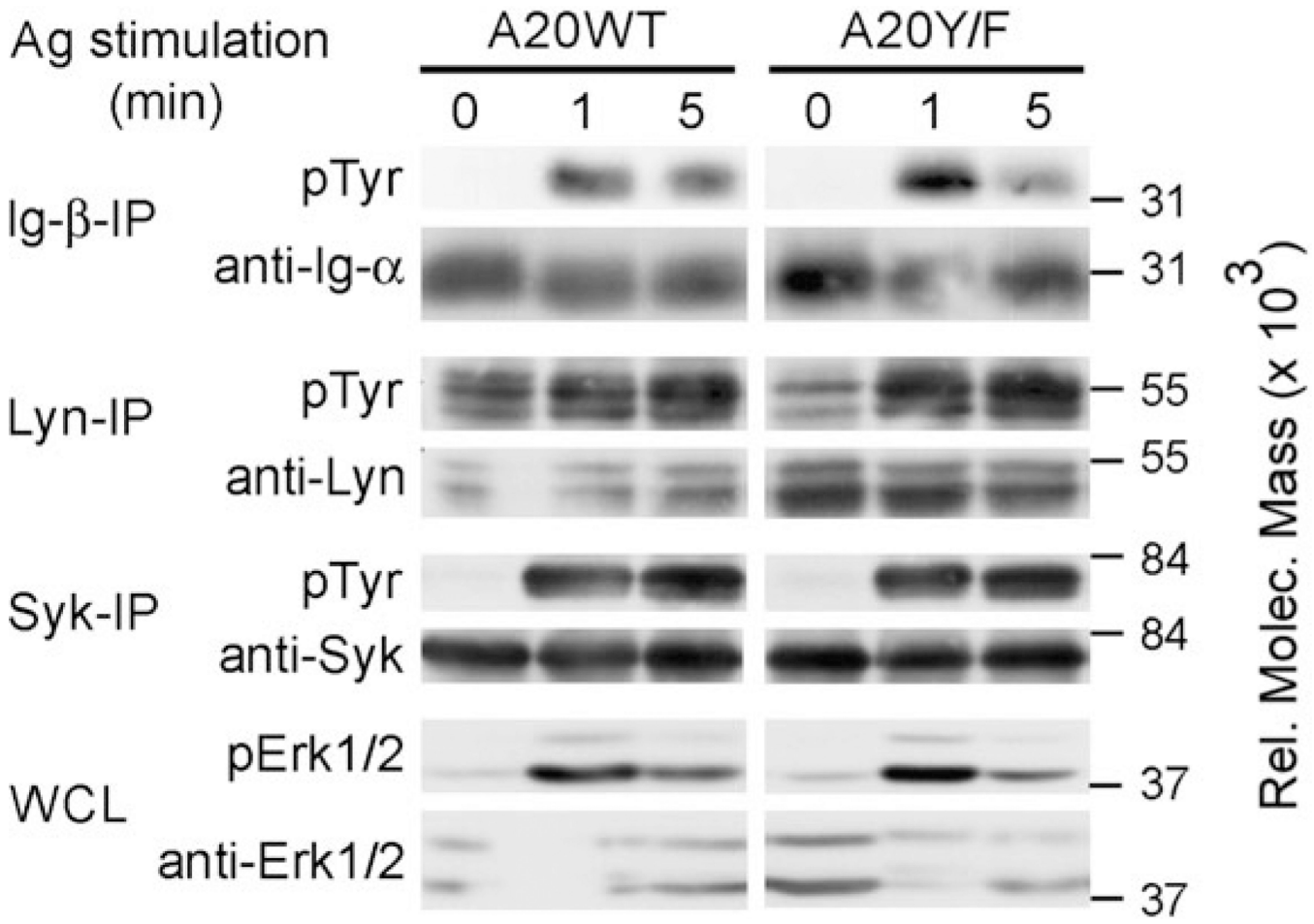

Fig. 3.

A20Y/F cells induce BCR-mediated signal transduction at a level comparable to A20WT cells at the times of receptor dissociation. A20WT and A20Y/F cells (10×10^6/IP, 5×10^6/WCL) were either unstimulated or antigen stimulated for the indicated times. Whole-cell lysates were blotted for Erk. Lyn, Ig-β and Syk were immunoprecipitated from cell lysates and blotted for phosphotyrosine and protein levels. The data are representative of three experiments.

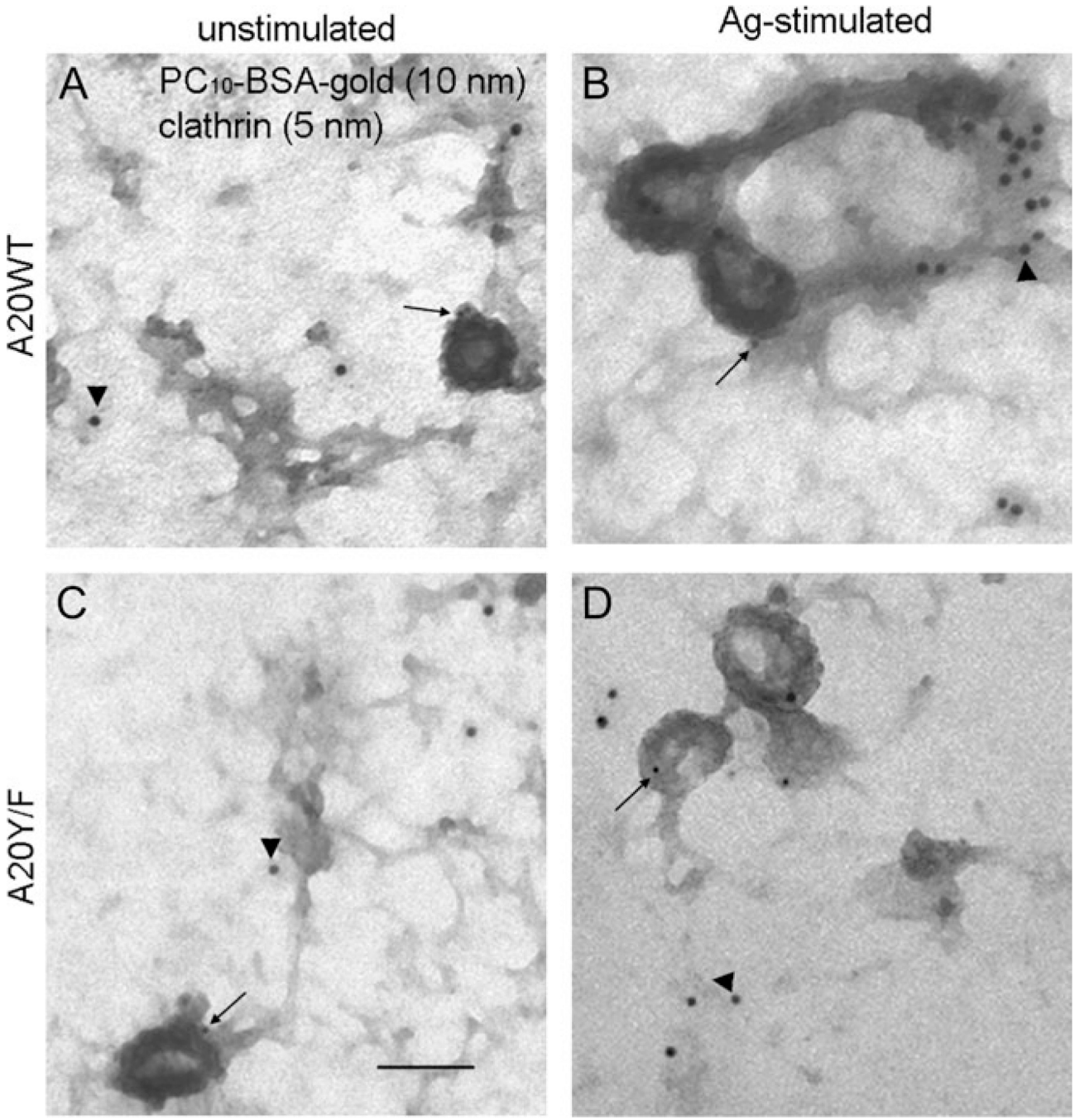

Fig. 4.

The association of antigen-bound μm with CCV requires the transmembrane tyrosine of μm. (A–D) Cells were either fixed prior to staining for μm or stimulated with PC_{10}–BSA–gold (10 nm) for 10 min. Native plasma membranes were prepared and stained for clathrin (5 nm). TEM images were taken at a magnification of ×25 000. The numbers of 10-nm gold particles are tabulated in Table 1 and represent data from 11 different native membrane sheets representing three experiments. Arrowheads and small arrows indicate antigen (10 nm) and clathrin (5 nm) staining, respectively. Bar = 100 nm.

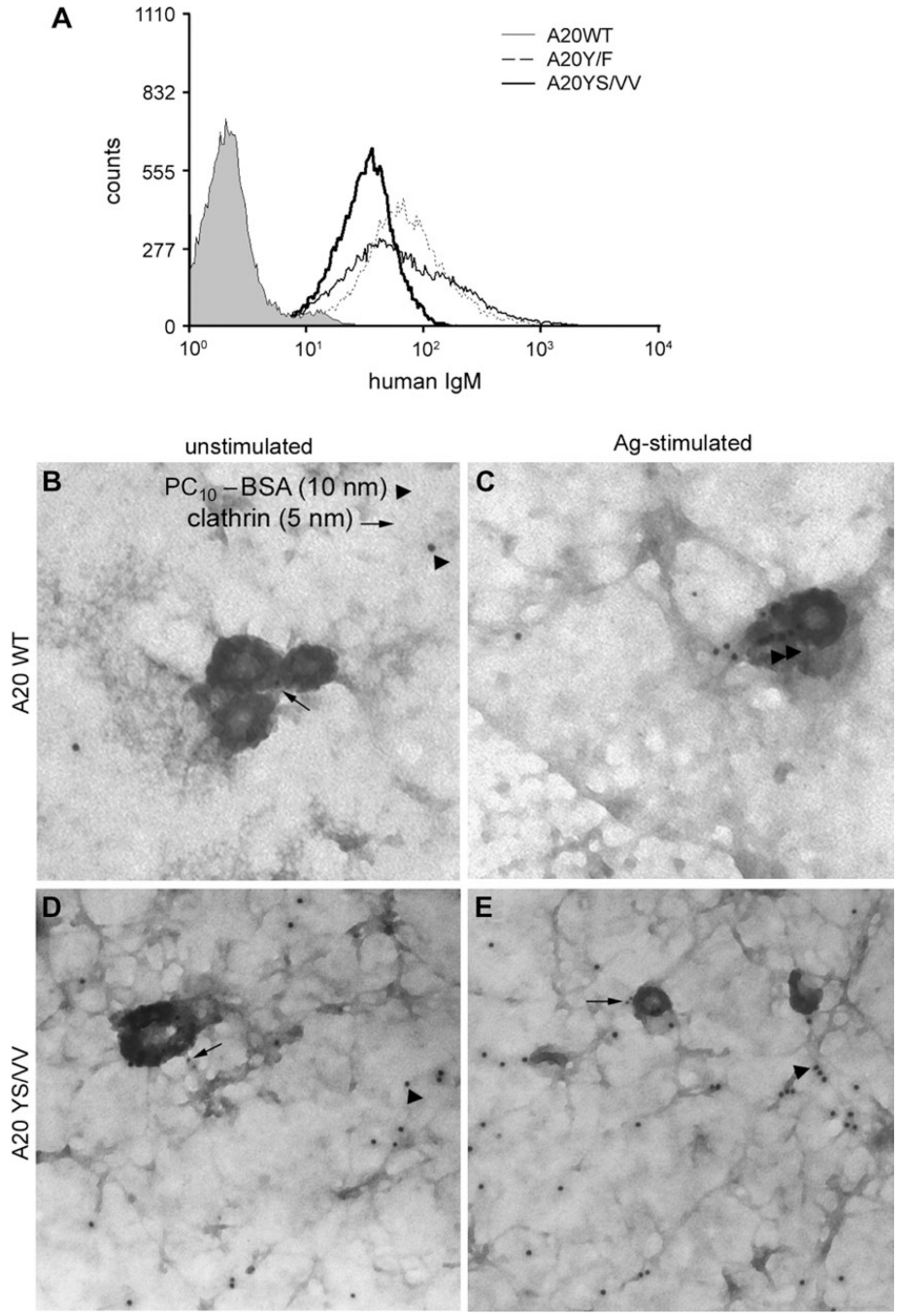

Fig. 5.

Exposure of the transmembrane region of μm is not sufficient to induce the association of receptors with CCVs. (A) A20WT, A20YS/VV and A20Y/F cells were stained with FITC-conjugated anti-human IgM and analyzed by flow cytometry. The parental cell line (A20) was used as negative control (filled histogram). (B–E) A20WT or A20YS/VV cells were either fixed prior to staining for μm or stimulated 5 min with PC_{10}–BSA– gold (10 nm, arrow heads). Native plasma membrane sheets were prepared and stained for clathrin (5 nm, small arrows). The numbers of 10-nm gold particles are tabulated in Table 2. The data represent five different native membrane sheets representing two experiments.

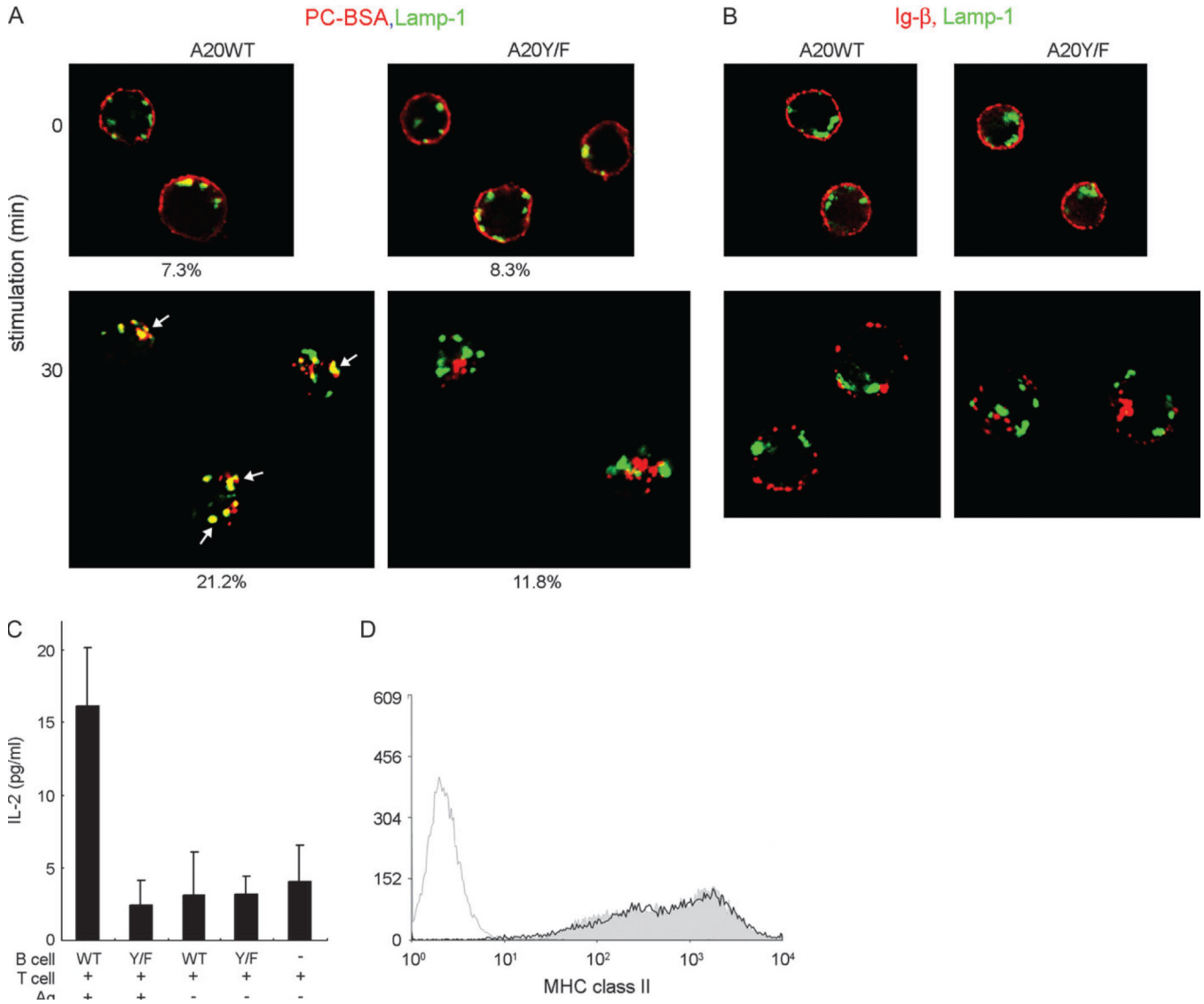

Fig. 6.

The transmembrane tyrosine of μm is required for antigen processing and presentation. (A–B) Cells were either fixed prior to staining or labeled on ice with fluorochrome-conjugated PC–BSA (A) or anti-Ig-β (B). After stimulation for 30 min, cells were fixed, permeabilized and stained for LAMP-1. Arrows indicate the co-localization of antigen and LAMP-1 and their percent co-localization is shown (A). The data were quantitated from 21 to 30 cells from three independent experiments. (C) A20WT (WT) and A20Y/F (Y/F) were pulsed with antigen (PC_3–OVA; 30 μg ml^{-1}) for 18 h, fixed and cultured with DO11.10 T cells for 24 h. The amount of IL-2 secreted by the OVA-specific T cells was measured by ELISA. The data are from four experiments. (D) A20WT (solid line) and A20Y/F (shaded) cells were stained with FITC-conjugated anti-mouse MHC class II (M5/ 114) antibody. Cells stained with isotype control antibody are shown as a gray line.

Table 1

Assessment of subcellular localization of μm, Ig-α and CCVs on native membrane sheets in A20WT and A20Y/F cells

	A20WT			A20Y/F		
Time (minutes)[a]	0	5	10	0	5	10
Total PC_{10}–BSA–gold (10 nm)[b]	2722	4145	2577	1928	3578	3189
Co-localization with Ig-α (5 m)	51%	25%	29%	52%	53%	54%
Time (minutes)	0	5	10	0	5	10
Total PC_{10}–BSA–gold (10 nm)[c]	1494	4225	2861	1365	2806	2847
Co-localization with clathrin (5 nm)	7%	27%	24%	8%	10%	11%

Cells were either fixed prior to μm staining ($t = 0$) or stimulated with PC_{10}–BSA–gold (10 nm). Native membrane sheets were prepared and stained on the intracellular side for Ig-α (5 nm) or clathrin (5 nm). Membrane localization of gold particles was analyzed by TEM at ×25 000.

[a] Time = 0 samples were either fixed at room temperature or held on ice while being labeled for μm. No difference between the two methods in the co-localization of μm with Ig-α was noted.

[b] Gold particles (10 nm) were counted from 12 to 14 different native membrane sheets obtained from three different experiments.

[c] Gold particles (10 nm) were counted from 11–21 different native membrane sheets obtained from three different experiments.

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Table 2

Assessment of subcellular localization of μm and CCVs on native membrane sheets in A20WT and A20YS/VV cells

	A20WT		A20YS/VV	
Time (minutes)[a]	0	5	0	5
Total PC_{10}–BSA–gold (10 nm)[b]	1494	4225	1364	1740
Co-localization with clathrin (5 nm)	7%	27%	10%	11%

Cells were either fixed prior to μm staining ($t = 0$) or stimulated with PC_{10}–BSA–gold (10 nm). Native membrane sheets were prepared and stained on the intracellular side for clathrin (5 nm). Membrane localization of gold particles was analyzed by TEM at ×25 000.

[a] Time = 0 samples were either fixed at room temperature or held on ice while being labeled for μm.

[b] Gold particles (10 nm) were counted from 11 to 21 different native membrane sheets obtained from three different experiments.